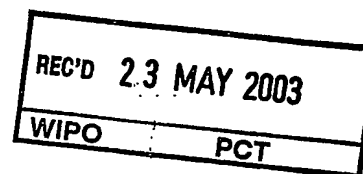


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08 April 2003

Maiken Lind

20 JUNI 2002

Title

An improved method for synthesising templated molecules

PVS**Technical Field of the Invention**

5 The present invention relates to a method for synthesising templated molecules. The method implies a high local concentration of reactive groups intended to participate in a formation of a linkage, thus increasing the probability of linkage formation. The invention also relates to a library, that is a plurality of templated molecules, wherein each of the templated molecules are attached to the template
10 which directed the synthesis thereof.

Background

The generation of molecules carrying new properties remains a challenging task. Recently, a number of procedures have been suggested that should allow a more
15 efficient generation and screening of a larger number of molecules. The approaches taken involve the encoding and/or templating of molecules other than natural biopolymers such as peptide, RNA and DNA. These approaches allow the researcher to generate and screen a huge number of molecules in a short time. This should lead to better molecules carrying the desired properties.

20 The central dogma of biology describes the one-way flow of information from DNA to RNA to protein. Recently, methods such as phage display, peptides-on-plasmids, ribosome display and mRNA-protein fusion have been developed, allowing the transfer of information from the level of protein/peptide to RNA or DNA. This has
25 enabled the use of molecular evolution to be applied on huge numbers of peptides that are exposed to an enrichment process, where after the enriched pool of molecules (enriched for a particular feature, such as binding to receptor protein) are amplified, by exploiting information flow from the peptide to DNA and then amplifying the DNA.

30 More recently, approaches have been developed that allow the encoding of polypeptides and other biochemical polymers. An example of this approach is disclosed in US 5,723,598, which pertains to the identification of a biochemical polymer that participates in a preselected binding interaction with a target to form a binding reaction complex. The prior art method encompasses the generation of a library of bi-
35

functional molecules. One part of the bifunctional molecule is the biochemical polymer and the other part is an identifier oligonucleotide comprising a sequence of nucleotides which encodes and identifies the biochemical polymer. Following the generation of the library of the bifunctional molecules, a partitioning with respect to affinity towards the target is conducted and the identifier oligonucleotide part of the bifunctional molecule is amplified by means of PCR. Eventually, the PCR amplicons are sequenced and decoded for identification of the biochemical polymer. This approach does not, however, allow one-pot amplification of the library members. Furthermore, the sequence of nucleotides serves to identify the biochemical molecule only after a laborious sequencing process. Thus the flow of information from the identifier sequence to the biochemical polymer is restrained.

Halpin and Harbury have in WO 00/23458 suggested an improvement to the approach stipulated immediately above, wherein the molecules formed are not only identified but also directed by the nucleic acid tag. The approach is based on the traditional split-and-combine strategy for synthesis of combinatorial libraries comprising two or more synthetic steps. Plurality nucleic acid templates are used, each having at one end a chemical reactive site and dispersed throughout the stand a plurality of codons regions, each of said codon regions in turn specifying different codons. Separately, each of the strands, identified by a first codon region, is reacted at the chemical reaction sites with specific selected reagents. Subsequently, all the strands are pooled and subjected to a second partitioning based on a second codon region. The split-and-combine method is conducted an appropriate number of times to produce a library of typically between 10^3 and 10^6 different compounds. The split-and-combine method is cumbersome and generates only a relatively small library.

Specifically, the present invention aims at suggesting a solution for increasing the local concentration of reactants to increase the probability of a reaction.

Summary of the Invention

The present invention provides a method for synthesising a templated molecule, said method comprising the steps of:

1. A method for synthesising a templated molecule, comprising the steps of:
 - a) providing at least one template comprising of one or more codons,

- b) providing a first functional entity attached to a zipping domain, said zipping domain comprises a first part of a molecule pair, being capable of reversible interaction with a second part of the molecule pair,
 - c) providing one or more building blocks, each comprising an anti-codon, a further functional entity and a linker connecting the anti-codon and the functional entity, wherein the anti-codon complements a codon of a template, the functional entity is connected to a zipping domain comprising the second part of said molecule pair, and is capable of being chemically connected to the first functional entity,
 - d) contacting the components of step a), b), and c) with each other under conditions allowing specific hybridisation of the anti-codon(s) to the codon(s) of the template(s) and dimerization of the two parts of the molecule pair,
 - e) allowing the functional entity of the building block to form a chemical connection to the first functional entity,
 - f) optionally, cleaving one or more linkers, provided that at least one linker remains to connect the functional entities with the template,
 - g) obtaining a templated molecule attached to the template which directed the synthesis thereof.
- The template comprises in a preferred embodiment two or more codons, such as three to fifteen codons. The first functional entity can then be connected to two or more functional entities. The method may be conducted only once to connect the scaffold functional entity with the desired amount of functional entities or the steps of d) through f) may be repeated one or more times to sequentially add building blocks harbouring functional entities to be attached to a functional entity.
- The zipper box dimerization domains may be characterized as a pair of molecular moieties comprising a zipper box when the two interacting moieties are able to reversibly dimerize in an ordered way, thereby bringing reactive groups attached to them into close proximity. Reversibility is required in a preferred aspect in order to allow different dimerization domains to interact at different times with the same zipper domain. Many types of molecular moieties may be employed as zipper domains, of which here follows a non-comprehensive list of appropriate pairs of zipper domains: i) DNA/DNA, DNA/RNA, LNA/DNA, PNA/RNA, various combinations of nucleotides and nucleotide analogs; ii) peptide/peptide, e.g. base and acid leucine

zipper (coiled coil structure of two alpha-helices), antibody/antigen; iii) nucleic acid-peptide, e.g. Zinck-finger DNA binding domain/dsDNA; iv) peptide/small organic molecule, e.g., streptavidin/biotin; v) small organic molecule/small organic molecule, e.g., nitrilotriacetic acid (NTA)/nitrilotriacetic acid (NTA)-Zn⁺⁺; vi) positively charged moiety/negatively charged moiety, e.g., polyglutamic acid/polylysine.

The zipper box can be chosen according to the conditions of the reaction that it is supposed to enhance. For example, if the reaction is performed at moderate temperature and at reasonably high salt concentration, DNA/DNA zipper boxes may be used. By varying the length of the zipper box (the complementary DNA strands), one may design zipper boxes of desired stability and dynamics. Other types of zipper boxes will be very dependent on pH. For example, the interaction strength and dynamics of a glutamate/lysine pair will be very dependent on pH, as for example the polyglutamate will be highly negatively charged at high pH, and not charged at all at low pH.

The functional entity is preferably attached to the template through one or more covalent links. However, it may be appropriate that the first functional entity is connected to a sequence of nucleic acids complementing a sequence of nucleic acids harboured by the template to enable the attachment of a scaffold to the template by hybridisation. In this way it will be possible to encode several different scaffolds by the template. In a preferred embodiment of the invention, the first functional entity is a scaffold, i.e. a chemical moiety which is amended, usually by addition of functional groups emanating from one or more building blocks.

The zipping domain may be placed relative to the first functional entity of the regular entity in any way that promote the proximity of the functional entities. In a preferred aspect of the invention the zipping domain is a part of the linker of the building block. Preferably, the zipping domain is proximal to the functional entity. Still more preferred the zipping domain is spaced from the functional entity with no more than 2 nucleic acids monomers. Also, it is preferred that the zipping domain and the functional entities are spaced by no more than 2 nucleic acid monomers. In a most preferred embodiment, the zipping domain of the functional entity of the building block and the first functional entity is distanced from the respective entities with the same number of nucleic acid monomers to provide for a high local

concentration of functional entities. The distance of the zipping domain of the functional entity of the building block and the first functional entity, respectively, to the functional entities are preferably zero nucleotide monomers. In other words, it is preferred that the two functional entities intended to form a connection is attached to the terminal nucleotide of the zipping domain.

The desired number of the nucleic acid monomers of the zipping domain depend largely on the temperature and stringency conditions in general used during the synthesis. If a low stringency and/or a relatively low temperature is preferred the number of nucleic acid monomers may be as low as 3. However, a low number of nucleic acid monomers in the sequence of the zipper domain may increase the risk of hybridisation to e.g. the template or building blocks. It is therefore, in general, preferred to use at least 4 nucleic acid monomers. According to a preferred embodiment of the invention the zipping domain sequence comprises 3 to 20 nucleic acid monomers. In a still more preferred embodiment the zipping domain sequence comprises 4 to 16 nucleic acid monomers. Most preferred is a zipping domain sequence comprising 5 to 10 nucleic acid monomers.

In a preferred aspect of the invention, the annealing temperature of the codon:anti-codon hybrid is higher than the annealing temperature of the zipping domain hybrid to ensure that the building block remain attached to the template even though the interaction of the zipping domains is eliminated. The above aspect is specifically preferred when the contacting according to step d) is performed by alternating the temperature below and above the annealing temperature of the zipping domain. The effect of the alternation is increased when the alternating is performed a plurality of times.

To avoid the release of the building block from the template, the highest alternating temperature is preferably below the annealing temperature of the codon:anti-codon hybrid.

According to a preferred aspect of the invention, when the template comprises two or more codons the building blocks attached to these codons have essentially identical sequences of the zipping domain. An alternation of the temperature will then attract the different functional entities annealed through building blocks to the

scaffold. Thus, it is possible to have a variety of functional entities brought into close proximity of the scaffold.

5 In an aspect of the invention the method is used to generate a library of templated molecules attached to the template (or, alternatively, a complementing template) which directed the synthesis of the molecule. As an example, a library may be generated by having more than one possible codon:anti-codon interaction. This may be conducted by having several building blocks with different functional entities but similar anti-codons. However, to obtain a one-to-one relationship between the
10 identity of the functional entity connected to the scaffold and the codon of the template, it is usually preferred that each building block carries a specific anti-codon which identify the functional entity.

15 A library preferably comprises a plurality of templates with different unique codons and/or order of unique codons. A plurality of building blocks having anti-codons corresponding to the unique codons of the templates is usually provided. In one aspect of the invention, a specific building block is provided for each of the unique codons. In another aspect some of the codons are not matched by a building block or alternatively blocked by a oligonucleotide sequence without a functional entity.

20 In the following the principle is illustrated for a specific non-limitating example. The anti-codons in this example are approximately 20 nucleotides long (and has a melting temperature towards its complementary sequence of approximately 60 °C), whereas the zipper domain is approximately 5 nucleotides long (and has a much
25 lower melting temperature, e.g. around 17 °C). The building blocks and the plurality of templates are incubated together, at a medium temperature (e.g., 55 °C), allowing the anti-codons to find and bind to the corresponding codons. At this temperature, the anti-codons interact efficiently and specifically with the codons, whereas the zipper boxes do not interact efficiently. Excess un-bound building blocks are washed
30 away. Then the reactions between reactive groups of neighbouring functional entities are initiated by lowering the temperature to e.g. 10 °C, and potentially changing conditions other than the temperature. At 10 °C the zipper domains of the regular building blocks will interact with the complementary sequence of the zipper domain of the scaffold functional entity, thereby bringing the reactive groups into very close
35 proximity (see figure 14). This increases the local concentration of the reactive

groups significantly, and as a result the reactive groups react. Then again, the temperature is increased to the medium temperature (55 °C) and the zipping box is melted resulting in a separation of the functional entities. When the temperature subsequently is decreased to about 10 °C, another building block may hybridize its zipper domain to the zipper domain of the scaffold, whereafter its functional entity may react with the scaffold.

Brief Description of the Figures

The following figures are referred to in this description:

Fig. 1 shows a reproduction of a PAGE gel displaying cross-linking of amino functionalities of two oligonucleotides annealed to a common template.

Fig. 2 shows a reproduction of a PAGE gel showing two oligonucleotides annealed to common template and cross-linked with a spacing of 0, 1, 2, and 30 base pair.

Fig. 3 shows a reproduction of a PAGE gel displaying cross-linking of two oligonucleotides terminated with a amine and carboxylic acid, respectively.

Fig. 4 shows a reproduction of a PAGE gel showing the influence of different pH profiles on cross-linking efficiency.

Fig. 5 shows a reproduction of a PAGE gel showing the influence of different pH profiles on cross-linking efficiency.

Fig. 6 shows a reproduction of a PAGE gel displaying cross-linking efficiency at pH 9.

Fig. 7 shows a reproduction of a PAGE gel displaying cross-linking efficiency at pH 10.

Fig. 8 shows a reproduction of a PAGE gel analysing the effect of absence of template when a 10 mer zipper box is used.

Fig. 9 shows a reproduction of a PAGE gel analysing the effect higher incubation temperature on the cross-linking efficiency.

Fig. 10 shows an image of a PAGE gel displaying the effect of a 5 mer zipper box on the cross-linking efficiency.

Fig. 11 shows an image of a PAGE gel displaying the effect of different temperatures on the cross-linking efficiency when a 10 mer zipper box is used.

Fig. 12 shows a schematic drawing of the general principle used in the experiments.

Fig. 13 shows a schematic drawing of the use of a dimerisation domain in the synthesis of (A) a scaffolded molecule and (B) a polymeric molecule.

Fig. 14 shows a preferred embodiment of the general principle.

5 Fig. 15 shows a LC-chromatogram of the transfer of two identical functional entities to a scaffold molecule.

10 In figure 13, a schematic drawing of the use of a dimerisation domain in the synthesis of (A) a scaffolded molecule and (B) a polymeric molecule is showed. When templating a scaffolded molecule (containing in this example four reactive groups of the same kind, Y), it is convenient to use four building blocks with identical zipper boxes ("b"), and one building block (carrying the four reactive groups Y) with a zipper box ("a") that is complementary to ("b"). When templating a polymeric molecule one may alternate between the zipper identity, i.e. first building block carries a zipper box ("a"), second building block in the array carries ("b") that dimerize with ("a"),
15 third building block carries ("a"), etc.

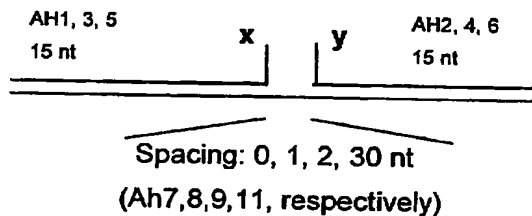
The preferred embodiment shown in Fig. 14 increases the local concentration of the reactive groups X and Y, by bringing X and Y into closer proximity through the dimerization of two zipper boxes. In this example, three building blocks are shown,
20 each carrying a zipper box, two of which are the same sequence ("a") and one is the complementary sequence ("b"). First, the building blocks are annealed to the template at a medium temperature (where the interaction between the zipper boxes is insignificant). Then the temperature is decreased to a lower temperature where two complementary zipper boxes ("a" (of the first building block) and "b" (of the second building block)) anneal to each other. This brings X and Y into close proximity, and X and Y may react to form YX. In the example, the reaction between X and Y involves a transfer of X from the first building block to the second building block carrying Y. When the temperature is increased to a medium temperature the zipper boxes dissociate. When the temperature is then lowered the zipper box of the second building block may anneal to the zipper box of the third building block (which carries a reactive group X). As a result, this X may now be transferred to the second building block, as a result of the increased proximity and hence increased reactivity between X and Y.
30

Examples

- 5 Here follows first methods and materials used, data obtained, and finally the data are discussed.

General methods and materials

- 10 In order to examine the reaction efficiency between two reactive groups, each coupled to a oligonucleotide, when the two oligos are annealed to neighbouring sites on the same template, the general set-up shown immediately below was used. The two oligos contain terminal nucleotides (X, Y, and Z) derivatized with a carboxylic acid or an amine, as described below the figure. After reaction ("cross-linking") of the reactive groups on the termini of the two oligos, the cross-linking efficiency may be analyzed by polyacrylamide gel electrophoresis, as the two oligos become coupled as a result of this cross-linking, and therefore migrate slower through the column.



Building blocks:

- 20
- Ah 1: 5'- GCTACTCGTACGAGX
 - Ah 3: 5'- GCTACTCGTACGAGY
 - Ah 5: 5'- GCTACTCGTACGAGZ
 - Ah 2: 5'- XCACTTGCAGACAGC
 - Ah 4: 5'- YCACTTGCAGACAGC
- 25
- Ah 6: 5'- ZCACTTGCAGACAGC
 - Ah 14: 5'-GCTACTCGTACGAG
 - Ah 23: 5'- GCTACTGGCATCGGX
 - Ah 24:5'- GCTACTGGCATCGGY
 - Ah 27:5'- YCACTTGCAGACAGC

30

In examples pertaining to a zipper box the following sequences was used

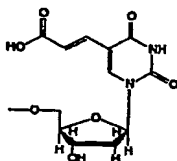
- AH36: 5'-CGACCTCTGGATTGCATCGGTCATGGCTGACTGTCCGTCGAA-TGTGTCCAGTTACX
- 5 • AH37: 5'-ZGTAACTGGACTGTAAGCTGCCTGTCAGTCGGTACTGACCT-GTCGAGCATCCAGCT
- AH51: 5'-ZGTAACACCTGTGTAAGCTGCCTGTCAGTCGGTACTGACCT-GTCGAGCATCCAGCT
- AH67: 5'- ZCATTGACCTGTGTAAGCTGCCTGTCAGTCGGTACTG-ACCTGTCGAGCATCCAGCT
- 10 • AH69: 5'- AGZAACACCTGTGTAAGCTGCCTGTCAGTCGGTACTG-ACCTGTCGAGCATCCAGCT
- AH66: 5'-ZTTGTAACTGGACTGTAAGCTGCCTGTCAGTCGGTACTGACC-TGTCGAGCATCCAGCT
- 15 • AH65: 5'-CGACCTCTGGATTGCATCGGTCATGGCTGACTGTCCGTCG-AATGTGTCCAGTTACTTX

Zipper box sequences are underlined.

- X= Carboxy-dT
- 20 • Y= Amino-Modifier C2 dT
- Z= Amino-Modifier C6 dT

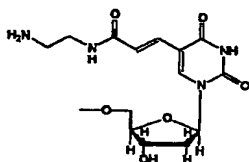
Carboxy-modifier C2 dT

25



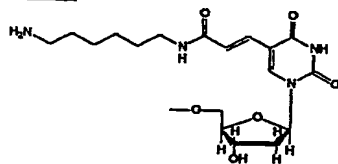
Amino modifier C2 dT

30



Amino modifier C6 dT

35



The oligonucleotides were prepared following the conventional phosphoramidite approach. X was incorporated using the commercially available carboxy-dT phosphoramidite (10-1035-90 from Glen research). The oligonucleotides terminated with Y and Z can be prepared from the corresponding X terminated oligonucleotides using the general procedure:

Templates:

Ah 28: 5'-GCTGTCTGCAAGTGAACCGATGCCAGTAGC
 Ah 38: 5'- AGCTGGATGCTCGACAGGTCCCGATGCAATCCAGAGG TCG

Ah7: 5'-
 GCTGTCTGCAAGTGAACCTCGTACGAGTAGCGACAGTCGACATCGGTCACG
 -biotin-3'

Ah 8: 5'-
 GCTGTCTGCAAGTGACACTCGTACGAGTAGCGACAGTCGACATCGGTCAC
 G-biotin-3'

Ah 9: 5'-
 GCTGTCTGCAAGTGACGACTCGTACGAGTAGCGACAGTCGACATCGGTCA
 CG-biotin-3'

Ah 11: 5'-
 GCTGTCTGCAAGTGACGACTGATCCAGTGACATGCGTACCATCGAACTCG
 TACGAGTAGCGACAGTCGACATCGGTCACG-biotin-3'

The templates was prepared by conventional phosphoramidate synthesis.

Buffers.

Buffer A (100 mM Hepes pH= 7,5, 1 M NaCl)
 Buffer B: (100 mM NaPO₄ pH=6, 1 M NaCl)
 Buffer C: (100 mM NaBorate pH=9, 1 M NaCl)
 Buffer D: (100 mM NaBorate pH=10, 1 M NaCl)
 Buffer E: (500 mM NaPO₄ pH=7, 1 M NaCl)
 Buffer F: (500 mM NaPO₄ pH=8, 1 M NaCl)

Annealing of DNA oligonucleotides.

Mix oligos in relevant buffer and heat at 80° C then cool to 28° C (-2° C/30 sek).

5'-Labeling with ^{32}P .

- 5 Mix 200 pmol oligonucleotide, 2 μl 10 x phosphorylation buffer (Promega cat#4103),
1 μl T4 Polynucleotid Kinase (Promega cat#4103), 1 μl γ - ^{32}P ATP, H_2O ad 20 μl .
Incubate at 37° C , 10-30 minutes.

PAGE (polyacrylamide gel electrophoresis).

- 10 The samples are mixed with formamide dye 1:1 (98% formamide, 10 mM EDTA, pH 8, 0,025 % Xylene Cyanol, 0,025% Bromphenol Blue), incubate at 80° C for 2 minutes, and run on a denaturing 10% polyacrylamide gel. Develop gel using autoradiography (Kodak, BioMax film).

Example 1.

- 15 Mix 2 μl Buffer A, 2 μl relevant oligo 1 (2 pmol/ μl), 2 μl relevant oligo 2 (2 pmol/ μl), 4 μl relevant oligo 3 (2 pmol/ μl) (See table I, below).

Table I:

Experiment	Oligo 1 (^{32}P -labelled)	Oligo 2	Oligo 3
A	Ah 3	Ah 4	Ah 7
B	Ah 5	Ah 6	Ah 7
C	Ah 5	Ah 6	None
D	Ah 5	Ah 6	Ah 8
E	Ah 5	Ah 6	Ah 9
F	Ah 14	Ah 6	Ah 7

20

Anneal as described above. Add 1 μl 100 mM, 1 μl 10 mM, or 0,1 μl 10 mM TSAT (Tris-succinimidyl aminotriacetate, Pierce cat#33063 dissolved in DMSO). Incubate at 25° C for about 1h, then analyze by 10% urea polyacrylamide gel electrophoresis.

- 25 The results are shown in Fig. 1.

Example 2

Mix 2 μ l Buffer A, 2 μ l relevant oligo 1 (0,2 pmol/ μ l), 1 μ l relevant oligo 2 (10 pmol/ μ l), 1 μ l relevant oligo 3 (10 pmol/ μ l), 4 μ l H₂O. (See table II, below)

Table II:

Experiment	Oligo 1 (³² P-labelled)	Oligo 2	Oligo 3
G	Ah5	Ah6	None
H	Ah5	Ah6	Ah7
I	Ah5	Ah6	Ah8
J	Ah5	Ah6	Ah9
K	Ah5	Ah6	Ah11

Anneal as described above. Add 1 µl 100 mM, 10 mM or 1 mM TSAT (Tris-succinimidyl aminotriacetate, Pierce cat#33063 dissolved in DMSO). Incubate at 25° C for about 5 h, then run 10% urea polyacrylamide gel, as described above.

The results are shown in Fig. 2

Example 3:

Mix 2 µl Buffer A, 2 µl relevant oligo 1 (0,2 pmol/ul), 1 µl relevant oligo 2 (10 pmol/ul), 1 µl relevant oligo 3 (10 pmol/ul), 4 µl H₂O. (See table III, below)

Table III:

Experiment	Oligo 1 (³² P-labelled)	Oligo 2	Oligo 3
L	Ah 1	Ah 6	None
M	Ah 1	Ah 6	Ah 7
N	Ah 1	Ah 6	Ah 8
O	Ah 1	Ah 6	Ah 9
P	Ah 1	Ah 6	Ah 11

Anneal as described above. Add 1 µl 1M, 100 mM, 10 mM or 1 mM EDC (1-Ethyl-3-(3-dimethylaminopropyl) Carbodiimide Hydrochloride, Fluka #03450) and 1 µl 100 mM NHS (N-Hydroxysuccinimid) (Aldrich cat # 13,067-2). Incubation at 25° C for about 5 h, and analyze by 10% urea polyacrylamide gel electrophoresis, as described above.

The results are shown in Fig. 3.

Example 4:

Mix 2 µl buffer A, B, C, D, E or F, 2 µl relevant oligo 1 (0,2 pmol/ul), 1 µl relevant oligo 2 (10 pmol/ul), 1 µl relevant oligo 3 (10 pmol/ul), 4 µl H₂O. (See table IV, below)

Table IV:

Experiment	Oligo 1 (³² P-labelled)	Oligo 2	Oligo 3
Q	Ah 1	Ah 6	Ah 7
R	Ah 5	Ah 6	Ah 7

Anneal as described above. Experiment Q is added 1 µl 100 mM EDC and 1 µl 100 mM NHS. Experiment R is added 1 µl 100 mM TSAT. Incubate at 25° C for about 1,5 h, and then analyze by 10% urea polyacrylamide gel electrophoresis.

The results are shown in Fig. 4.

Example 5:

Mix 2 µl buffer A or D, 2 µl relevant oligo 1 (0,2 pmol/ul), 2 µl relevant oligo 2 (10 pmol/ul), 2 µl relevant oligo 3 (10 pmol/ul), 2 µl H₂O. (See table V, below).

Table V:

Experiment	Oligo 1 (³² P-labelled)	Oligo 2	Oligo 3
S	Ah 5	Ah 6	Ah 7
T	Ah 14	Ah 6	Ah 7

Anneal as described above. Add 1 µl 100 mM TSAT. Incubate at 25° C for about 1,5 h, and then analyze by 10% urea polyacrylamide gel electrophoresis.

The results are shown Fig. 5.

Example 6:

Mix 2 µl buffer A, B or D, 1 µl relevant oligo 1 (2 pmol/ul), 1 µl relevant oligo 2 (10 pmol/ul), 1 µl relevant oligo 3 (10 pmol/ul), 5 µl H₂O. (See table VI, below).

Table VI:

Experiment	Oligo 1 (³² P-labelled)	Oligo 2	Oligo 3
UA (Buffer A)	Ah 23	Ah 27	Ah 28
VA (Buffer A)	Ah 23	Ah 27	None
UB (Buffer B)	Ah 23	Ah 27	Ah 28
VB (Buffer B)	Ah 23	Ah 27	None
X (Buffer D)	Ah 24	Ah 27	Ah 28
Y (Buffer D)	Ah 24	Ah 27	None

5 Anneal as described above. Experiment U and V is added 1 µl 100 mM EDC and 1 µl 100 mM NHS, incubated for about 1 h at 24°C, and then added 2 µl buffer C, then incubated for 30 minutes at 24°C. Experiment X and Y is added 2 µl 50 mM TSAT. Incubate at 24° C for about 1.5 h, and then analyzed by 10% urea polyacrylamide gel electrophoresis, as described above.

10 The results are shown in Fig. 6.

Example 7:

15 Mix 2 µl first Buffer (See below) , 1 µl Ah 23 (2 pmol/ul), 1 µl Ah 27 (10 pmol/ul), 1 µl Ah28 (10 pmol/ul), 5 µl H₂O. Anneal as described above, then add 1 µl 100 mM NHS and 1 µl 1 M EDC, incubate for 30 minutes at 24°C, then add 3 µl second buffer (See below). Incubate for 40 minutes at 24°C, and then analyze by 10% urea polyacrylamide gel electrophoresis.

Table VII:

Experiments	First Buffer	Second Buffer
7-1	Buffer A	Buffer A
7-2	Buffer A	Buffer C
7-3	Buffer A	Buffer D
7-4	Buffer B	Buffer D
7-5	Buffer B	Buffer C

20

The results are shown in Fig. 7.

Example 8:

Mix 8-1: Mix 2 µl buffer B, 5 µl Ah36 (0,4 pmol/ul), 1 µl Ah37 (2 pmol/ul), 1 µl Ah38 (2 pmol/ul), 1 µl H₂O.

- 5 Mix 8-2: Mix 2 µl buffer B, 5 µl Ah36 (0,4 pmol/ul), 1 µl Ah37 (2 pmol/ul), 2 µl H₂O. Anneal by heating to 80° C, then cool to 44° C (-2°C/30 sek). Add 1 µl 100 mM NHS and 1 µl 1 M EDC. Incubate at indicated temperatures (see below) for 45 minutes, then add 2 µl Buffer D. Incubate for about 2 h, and then analyze by 10% urea polyacrylamide gel electrophoresis.

10

Incubation temperatures:

45 °C, 48,2 °C, 53,0 °C, 58,5 °C, 63,1 °C, 65,6 °C

The results are shown in Fig. 8.

15

Example 9:

Mix 9-1: Mix 2 µl buffer B, 1 µl Ah36 (2 pmol/ul), 1 µl Ah51 (2 pmol/ul), 1 µl Ah38(2 pmol/ul), 5 µl H₂O.

Mix 9-2: Mix 2 µl buffer B, 1 µl Ah36 (2 pmol/ul), 1 µl Ah51 (2 pmol/ul), 6 µl H₂O

- 20 Anneal by heating to 80°C, then cool to 35° C (-2°C/30 sek)(For temperatures 1 to 6), or heat to 80°C, then cool to 15°C (-2°C/30sek)(For temperatures 7 to 12).

Add 1 µl 100 mM NHS and 1 µl 1 M EDC. Incubate at indicated temperatures (see below) for 1 h, then add 2 µl Buffer D. Incubate for 1 h, , and then analyze by 10% urea polyacrylamide gel electrophoresis, as described above.

25

Incubation temperatures:

1) 34,9°C, 2) 36,3°C, 3) 40,3°C, 4) 45,7°C, 5) 51,0°C, 6) 55,77, 7) 14,9°C, 8) 17,8°C, 9) 22,7°C, 10) 28,3°C, 11) 31,0°C, 12) 36°C

30

Mix 9-3: Mix 2 µl buffer B, 0,5 µl Ah36 (2 pmol/ul), 1 µl Ah51 (2 pmol/ul), 1 µl Ah38(2 pmol/ul), 5,5 µl H₂O

Mix 9-4: Mix 2 µl buffer B, 0,5 µl Ah36 (2 pmol/ul), 1 µl Ah51 (2 pmol/ul), 6,5 µl H₂O
Anneal by heat at 80° C then cool to 5° C (-2°C/30 sek).

Add 1 μ l 100 mM NHS and 1 μ l 1 M EDC. Incubate at different temperatures (see below) for 1 h, then add 2 μ l Buffer D. Incubate for 1 h, , and then analyze by 10% urea polyacrylamide gel electrophoresis.

5 Incubation temperatures:

1) 5,9°C, 2) 9,9°C, 3) 12,6°C, 4) 18,3°C, 5) 23,3°C, 6) 27,9°C 7) 35,6°C, 8) 45,9°C

The results are shown in Fig. 9, A and B.

10 **Example 10.**

Mix 2 μ l bufferA, 1 μ l relevant oligo 1 (2 pmol/ μ l), 1 μ l relevant oligo 2 (10 pmol/ μ l), 1 μ l relevant oligo 3 (10 pmol/ μ l), 5 μ l H₂O. (See table below). Anneal as described above.

15 Add 1 μ l 100 mM NHS and 1 μ l 1 M EDC. Incubate at different temperatures 1) 7,7°C, 2) 15,4°C, 3) 21,0°C 4) 26,2°C for about 2 h, and 5) 10°C for 1 sec. and then 35°C for 1 sec. Repeat 99 times. Analyze by 10% urea polyacrylamide gel electrophoresis.

20 Table VIII:

Experiment	Oligo 1 (³² P)	Oligo 2	Oligo 3
10-1	Ah36	None	Ah38
10-2	Ah36	None	None
10-3	Ah36	Ah51	Ah38
10-4	Ah36	Ah51	None
10-5	Ah36	Ah67	Ah38
10-6	Ah36	Ah67	None
10-7	Ah36	Ah69	Ah38
10-8	Ah36	Ah69	None

The results are shown in Fig. 10 A and Fig. 10 B.

Example 11

Mix 2,5 µl buffer A, 1 µl relevant oligo 1 (2 pmol/ul), 1 µl relevant oligo 2 (10 pmol/ul), 1 µl relevant oligo 3 (10 pmol/ul), 4,5 µl H₂O. (See table below). Anneal by heating to 80°C and then cool to 30°C or 55°C. Add 1 µl 100 mM NHS and 1 µl 1 M EDC. Incubate at 30°C or 55°C. Then analyze by 10% urea polyacrylamide gel electrophoresis.

Table IX:

Experiment	Oligo 1 (³² P-labelled)	Oligo 2	Oligo 3
11-1	Ah36	Ah37	Ah38
11-2	Ah36	Ah37	None
11-3	Ah65	Ah66	Ah38
11-4	Ah65	Ah66	None
11-5	Ah36	Ah66	Ah38
11-6	Ah36	Ah66	None
11-7	Ah65	Ah37	Ah38
11-8	Ah65	Ah37	None

The results are shown in Fig. 11.

Discussion of the results of the Examples 1 to 11**Influence of linker length and spacing between the reactive groups on cross-linking efficiency.**

We first examined the effect of changing the length of the linker that connects the amine and the nucleotide. Oligos Ah3 and Ah5 contain an amine separated from the base of the nucleotide by seven and eleven bonds, respectively (called amino modifier C2 dT and amino modifier C6 dT, see formulae above). These oligos were annealed immediately next to oligo Ah 4 or Ah6 (carrying amino modifier C2 dT and amino modifier C6 dT, respectively), i.e., with a spacing between the two oligos of 0 base pairs.

As seen in figure 1, lanes A and B, the efficiency of cross-linking is approximately equal for either amino modifier.

In all the following experiments, the oligo Ah5 (containing amino modifier C6 dT) was used as the reactive group amine.

5

Next, the two oligos were annealed to templates with spacings of 0, 1, 2, and 30 base pairs between the two oligos, and the efficiency of cross-linking examined. First, cross-linking using TSAT (Tris-succinimidyl aminotriacetate, Pierce cat#33063 dissolved in DMSO) was investigated. When oligos Ah5 and Ah6 were used, the efficiency of the cross-linking reaction were highest with a spacing of 0 base pairs (figure 1, lanes B; figure 2, panel H), less efficient with a spacing of 1 base pairs (figure 1, lanes D; figure 2, panel I), and very inefficient with spacings of 2 and 30 base pairs (figure 1, lanes E and F; figure 2, panel J and K).

10

Second, cross-linking of an amine and a carboxylic acid was examined. In this experiment, EDC (1-Ethyl-3-(3-dimethylaminopropyl) Carbodiimide Hydrochloride and NHS (N-Hydroxysuccinimide) was added in order to crosslink the two reactive groups. When oligos Ah1 and Ah6 were used, the efficiency of cross-linking was again highest for the shortest spacing of zero base pairs (figure 3, panel M), relatively high for a spacing of one base pairs (figure 3, panel N), and modest and insignificant for spacings of 2 and 30 base pairs, respectively (figure 3, panel O and P).

15

20

Optimization of TSAT and EDC concentration.

The importance of TSAT concentration was tested by using the oligos Ah5 and Ah 6. A concentration of 1 or 10 mM TSAT leads to more efficient cross-linking than both 0.1 mM and 100 mM TSAT (figure 1 and 2). The lower cross-linking efficiency obtained when using the highest TSAT concentration (100 mM) may be explained by two TSAT molecules reacting with each of the neighbouring amines.

25

Next, the importance of EDC concentration was examined for cross-linking an oligo carrying an amine (Ah6) and an oligo carrying a carboxylic acid (Ah1). Previously, it has been found that NHS concentrations of about 10 mM provides the highest cross-linking efficiency when used together with EDC. As shown in figure 3, 100 mM EDC results in the highest cross-linking efficiency when compared to 0.1 mM, 1 mM and 10 mM EDC.

30

35

Optimization of pH for TSAT and EDC/NHS cross-linking reactions.

Next, we tested the influence of different pH profiles for cross-linking efficiency using either the EDC/NHS or TSAT reagents.

5 A pH of 10 provides the most efficient TSAT cross-linking of two amines (figure 4, panel R; figure 5, panel S). Oligos Ah5 and Ah6 were used in this study. In experiment 6 (figure 6) a cross-linking efficiency of 80% is obtained using pH 10, and a spacing of zero base pairs between amine-carrying oligos Ah24 and Ah27. In other experiments where the linker that separates the complementing element (the region of the oligo that anneals to the template) and the reactive group (amine or carboxylic acid) is much larger (e.g. figure 11 and 12), the cross-linking efficiency is much lower.

10 Oligos Ah1 and Ah6 were next used to examine the influence of different pH profiles on the the cross-linking efficiency using EDC/NHS. The constant pH that mediates the most efficient cross-linking is pH 7.5 (figure 4, panel Q). However, an even better cross-linking efficiency is obtained when the pH is initially kept at pH 6, and then increased to pH 9 (figure 6) or 10 (figure 7). In the latter two experiments, oligos Ah23 and Ah27 were used. Under those conditions, the cross-linking efficiency is almost 100%. Note, that in these experiments the linker that connects the reactive group and the complementing element is relatively short (e.g. 11 bonds for the Ah27).

Examination of cross-linking efficiency when using a zipper box sequence.

25 We next examined the cross-linking efficiency using oligos carrying reactive groups (amine or carboxylic acid) where the linker connecting the reactive group and the annealing region were approximately 25 nucleotides.

In a first experiment oligos Ah36 (carrying a carboxylic acid) and Ah67 (carrying an amine) were used. The template used (Ah38) anneals the two oligos immediately adjacent, i.e. with a spacing of zero base pairs.

30 Under the conditions of the experiment, less than 5% cross-linking efficiency is observed, and only at the highest tested temperature (figure 10, A and B, lanes 5). In order to improve the cross-linking efficiency, we introduced a so-called zipper box sequence at the 5'- and 3' end of oligos Ah67 and Ah36, respectively, the same termini that carries the reactive groups. The zipper-boxes are complementary se-

35

quences, and thus may bring the reactive groups of the two oligos into closer proximity. Two different lengths of zipper boxes were tested, namely a 10'mer zipper box (Ah37/Ah66, Ah37 forming a DNA duplex of 10 base pairs) and a 5'mer zipper box (forming a DNA duplex of 5 base pairs). See Fig. 12. Moreover, different designs of zipper boxes were tested, e.g. oligos in which the reactive group is attached immediately adjacent to the zipper box (Ah36, Ah37, Ah51), or placed two nucleotides upstream from the zipper box (Ah65, Ah66), or placed in the middle of the zipper box (Ah67).

We first tested the effect of the 5'mer zipper box on cross-linking efficiency. As can be seen, the 5'mer zipper box improves the cross-linking efficiency dramatically (figure 10, A and B, compare lanes 3 and lanes 5). Note that the template is absolutely required for cross-linking at all temperatures tested. The highest cross-linking efficiency is obtained when the temperature is cycled 99 times up and down between 10°C and 35°C (figure 10B). A high efficiency is also obtained when the temperature is kept constant at 21°C or 26°C (figure 10A and B, lanes 3). The cross-linking efficiency does not improve further at temperatures above 26°C (figure 9, A and B).

We next tested the efficiency of cross-linking in the 10'mer zipper box format. Oligos Ah36 and Ah37 were annealed to template Ah38, and the cross-linking efficiency examined at various temperatures. A surprisingly high degree of cross-linking in the absence of template was observed (figure 8, 45°C and 48.2°C). However, at temperatures above 58.5°C, no cross-linking is observed in the absence of template.

Next, the different locations of the reactive groups relative to the zipper box was tested. As shown in figure 10, A and B, lanes 7, the cross-linking efficiency decreases dramatically when one of the two reactive groups is located in the middle of the zipper box (i.e., the reactive group is attached to a nucleotide involved in DNA double helix formation; Ah67).

The location of the reactive groups relative to the zipper box was also tested in the context of the 10'mer zipper box. In this context, when both reactive groups are separated from the zipper box by two nucleotides (Ah65, Ah66), the efficiency of cross-linking is slightly decreased (figure 11, compare lanes 1 and 3). The cross-linking efficiency is not changed dramatically when different combinations of Ah65, Ah66, Ah36 and Ah37 are tested (i.e., when the reactive groups are placed immedi-

ately next to the zipper box, or two nucleotides upstream). Note that the template is not absolutely required at all temperatures in the context of the 10'mer zipper box. This template-independency is particularly pronounced at lower temperature (e.g., figure 11, 30°C).

5

Example 12: A method for transforming a carboxylic acid containing oligonucleotide to a trisamine scaffold building block

- 10 The following oligos containing a modified nucleobase, with a carboxylic acid moiety, were synthesised using the conventional phosphoramidite approach:

F: 5'-GAC CTG TCG AGC ATC CAG CTG TCC ACA ATG X

- 15 G: 5'-GAC CTG TCG AGC ATC CAG CTT CAT GGG AAT TCC TCG TCC ACA ATG X

H: 5'-GAC CTG TCG AGC ATC CAG CTT CAT GGG AAT TCC TCG TCC ACA ATG XT

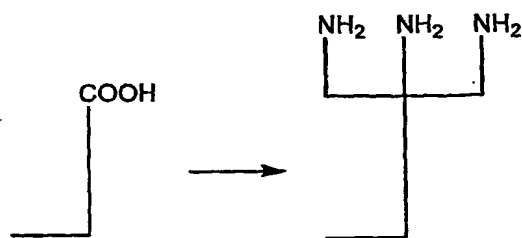
20

I: 5'-XGT AAC TGG AGG GTA AGC TCA TCC GAA TTC GGT ACT GAC CTG TCG AGC ATC CAG CT

- 25 X was incorporated using the commercially available carboxy-dT phosphoramidite (10-1035-90 from Glen research). The underlined nucleobases represent the zipper region.

30

Schematic representation of the reaction:



5 An oligo containing one modified nucleobase with a carboxylic acid moiety (1 nmol) was mixed with water (100 uL), hepes buffer (40 uL of a 200 mM, pH=7.5), NHS (20 uL of a 100 mM solution), EDC (20 uL of a freshly prepared 1 M solution) and a tetraamine (20 uL of a 100 mM solution). The reaction mixture was left o/n at room temperature. The volume was reduced to 60 uL by evaporation *in vacuo*. The pure
10 oligo was obtained by addition of NH₃ conc. (20 uL) followed by HPLC purification. It was possible to isolate a peak after approximately 6 min using the following gradient: : 0-3 minutes 100% A then 15% A and 85% B from 3-10 minutes then 100% B from 10-15 minutes then 100% A from 15-20 minutes. A = 2% acetonitrile in 10 mM TEAA and B = 80% acetonitrile in 10 mM TEAA.

15

Example 13: General procedure for attachment of a functional entity to a thio oligo.

The following oligo containing a modified nucleobase, with a S-triphenylmethyl protected thio moiety, was synthesised using the conventional phosphoramidite approach:
20

J: 5'-WCA TTG ACC TGT GTA AGC BTG CCT GTC AGT CGG TAC TCG ACC TCT GGA TTG CAT CGG

25 K: 5'-WCA TTG ACC TGT CTG CCB TGT CAG TCG GTA CTG TGG TAA CGC GGA TCG ACC T

L: 5'-WCA TTG ACC TGA ACC ATG BTA AGC TGC CTG TCA GTC GGT ACT ACG ACT ACG TTC AGG CAA GA

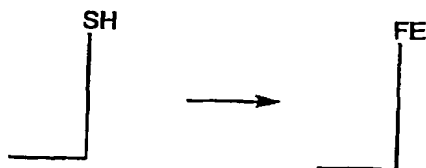
30

M: 5'-WCA TTG ACC TGA ACC ATG TBA AGC TGC CTG TCA GTC GGT ACT
TCA AGG ATC CAC GTG ACC AG

5 **W** was incorporated using the commercially available thiol modifier phosphoramidite (10-1926-90 from Glen research). B is an internal biotin incorporated using the commercially available phosphoramidite (10-1953-95 from Glen research). The nucleobases which are underlined and italic indicates the zipper region.

10 The S-triphenylmethyl protected thio oligo (10 nmol) was evaporated *in vacuo* and resuspended in TEAA buffer (200 uL of a 0.1M solution, pH=6.4). AgNO₃ (30 uL of a 1 M solution) was added and the mixture was left at room temperature for 1-2 hours. DTT (46 uL of a 1M solution) was added and left for 5-10 minutes. The reaction mixture was spun down (20.000 G for 20 minutes) and the supernatant was collected. The solid was extracted with additional TEAA buffer (100 ul of a 0.1 M solution, pH=6.4). The pure thio oligo was obtained by conventional EtOH-precipitation.

Schematic representation of the reaction:



20 The thio oligo (1 nmol) was dried *in vacuo* and treated with a building block comprising the functional entity (05087) in dimethylformamide (50 ul of a 0.1 M solution) and left o/n at rt. The thio oligo was spun down (20.000 G for 10 minutes) and the supernatant removed. Dimethylformamide (1 mL) was added and the loaded thio oligo was spun down (20.000 G for 10 minutes). The dimethylformamide was removed and the loaded thio oligo was resuspended in TEAA buffer (25 uL of a 0.1M solution, pH=6.4) and analysed by HPLC.

Example 14: General procedure for the templated synthesis of a scaffolded molecule, where the scaffold and two identical substituents are encoded by the template.

5

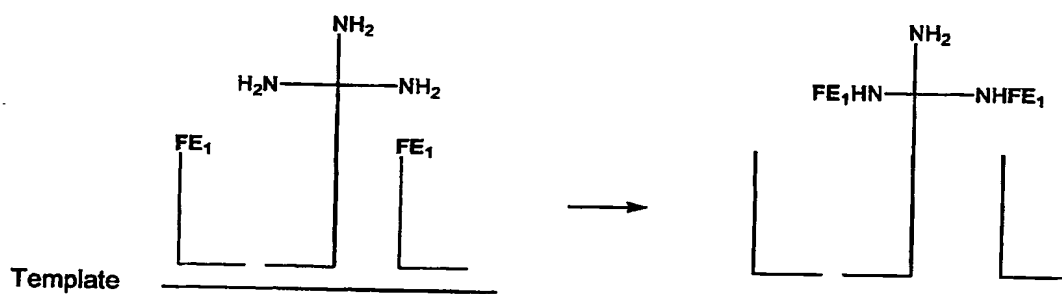
K: 5'-WCA TTG ACC TGT CTG CCB TGT CAG TCG GTA CTG TGG TAA CGC
GGA TCG ACC T

10

L: 5'-WCA TTG ACC TGA ACC ATG BTA AGC TGC CTG TCA GTC GGT ACT
ACG ACT ACG TTC AGG CAA GA

H: 5'-GAC CTG TCG AGC ATC CAG CTT CAT GGG AAT TCC TCG TCC ACA
ATG XT

15



20

25

The template oligo (1 nmol) was mixed with two thio oligos (K and L) loaded with the same functional entity (XXVI; 1 nmol) and the trisamine oligo H (1 nmol) in hepes-buffer (20 uL of a 100 mM hepes and 1 M NaCl solution, pH=7.5) and water (added to a final volume of 100 uL). The oligos were annealed to the template by heating to 50 °C and cooled (-2 °C/ 30 second) to 30 °C. The mixture was then left o/n at a fluctuating temperature (10 °C for 1 second then 35 °C for 1 second). The oligo complex was attached to streptavidine by addition of streptavidine beads (100 uL, prewashed with 2x1 mL 100 mM hepes buffer and 1M NaCl , pH=7.5). The beads were washed with hepes buffer (1mL). The trisamine scaffold oligo H was separated from the streptavidine bound complex by addition of water (200 uL) followed by

heating to 70 °C. The water was transferred and evaporated *in vacuo*, resuspended in TEAA buffer (45 uL of a 0.1 M solution) and product formation analysed by HPLC (see Figure 15).

- 5 The HPLC chromatogram shows the transfer of two functional entities to a scaffold oligo with three amino groups.

A) The top chromatogram shows the reference scaffold oligo G.

- 10 B) The bottom chromatogram show the streptavidine purified scaffold oligo G after the partial transfer of one (peak at 7.94 minutes) and two (peak at 10.76 minutes) identical functional entities (XXVI). The following gradient was used: 0-3 minutes 100% A then 15% A and 85% B from 3-10 minutes then 100% B from 10-15 minutes. A = 2% acetonitrile in 10 mM TEAA and B = 80% acetonitrile in 10 mM TEAA.

- 15 Due to the lipophilic nature of the functional entities a longer retention time, in the HPLC chromatogram, of the scaffolded molecule with two functional entities compared to one functional entity, was observed. The efficiency of the templated synthesis of a scaffolded molecule with the two identical functional entities (XXVI) was
- 20 about 25% (peak at 10.76 minutes in Figure 15).

Claims

1. A method for synthesising a templated molecule, comprising the steps of:
 - a) providing at least one template comprising of one or more codons,
 - 5 b) providing a first functional entity attached to a zipping domain, said zipping domain comprises a first part of a molecule pair being capable of reversible interaction with a second part of the molecule pair,
 - c) providing one or more building blocks, each comprising an anti-codon, a further functional entity and a linker connecting the anti-codon and the functional entity, wherein the anti-codon complements a codon of a template, the functional entity is connected to a zipping domain comprising the second part of said molecule pair, and is capable of being chemically connected to the first functional entity,
 - 10 d) contacting the components of step a), b), and c) with each other under conditions allowing specific hybridisation of the anti-codon(s) to the codon(s) of the template(s) and dimerization of the two parts of the molecule pair,
 - e) allowing the functional entity of the building block to form a chemical connection to the first functional entity,
 - 15 f) optionally, cleaving one or more linkers, provided that at least one linker remains to connect the functional entities with the template,
 - 20 g) obtaining a templated molecule attached to the template which directed the synthesis thereof.
2. The method according to claim 1, wherein step d) through f) is repeated one or more times.
- 25 3. The method according to claim 1 or 2, wherein the first functional entity is covalently connected to the template.
4. The method according to claim 1 or 2, wherein the first functional entity is part of a building block.
5. The method according to any of the preceding claims, wherein the molecule pair comprises two complementary sequences of nucleic acids or nucleic acid analogs.
- 30 6. The method according to claim 1 or 2, wherein the first functional entity is connected to a sequence of nucleic acids complementing a sequence of nucleic acids harboured by the template.

7. The method according to any of the preceding claims, wherein the zipping domain is a part of the linker of the building block.
8. The method according to claim 7, wherein the zipping domain is proximal to the functional entity.
- 5 9. The method according to claims 7 or 8, wherein the zipping domain is spaced from the functional entity with no more than 2 nucleic acids monomers.
- 10 10. The method according to claim 1, wherein the zipping domain and the functional entity of the scaffold is spaced by no more than 2 nucleic acid monomers.
11. The method according to any of the preceding claims, wherein the zipping domain of the functional entity of the building block and the scaffold functional entity is distanced from the respective entities with the same number of nucleic acid monomers.
12. The method according to any of the preceding claims, wherein the zipping domain sequence comprises 3 to 20 nucleic acid monomers.
- 15 13. The method according to claim 12, wherein the zipping domain sequence comprises 4 to 16 nucleic acid monomers.
14. The method according to claim 13, wherein the zipping domain sequence comprises 5 to 10 nucleic acid monomers.
- 20 15. The method according to any of the preceding claims, wherein the annealing temperature of the codon:anti-codon hybrid is higher than the annealing temperature of zipping domain hybrid.
16. The method according to any of the preceding claims, wherein the contacting according to step d) is performed by alternating the temperature below and above the annealing temperature of the zipping domain.
- 25 17. The method according to claim 16, wherein the alternating is performed a plurality of times.
18. The method according to claim 16 or 17, wherein the highest alternating temperature is below the annealing temperature of the codon:anti-codon hybrid.
19. A templated molecule obtainable according to any of the claim 1 to 18.

Abstract

5 The invention relates to a method for synthesising templated molecules attached to the templated which directed the synthesis thereof. The method involves a template, a scaffold functional entity and a functional entity attached to a building block, which, in turn, is attached the template. The scaffold functional entity and the functional entity of the building block are both provided with complementary dimerization domains allowing the functional entities to come into close proximity when the complementary domains interact with to each other. The method may be used for generating libraries of templated molecules which may be selected for biological activity.

0,1 mM TSAT 1 mM TSAT 10 mM TSAT

AB C0 DEF AB C0 DEF A B C 0 D E F

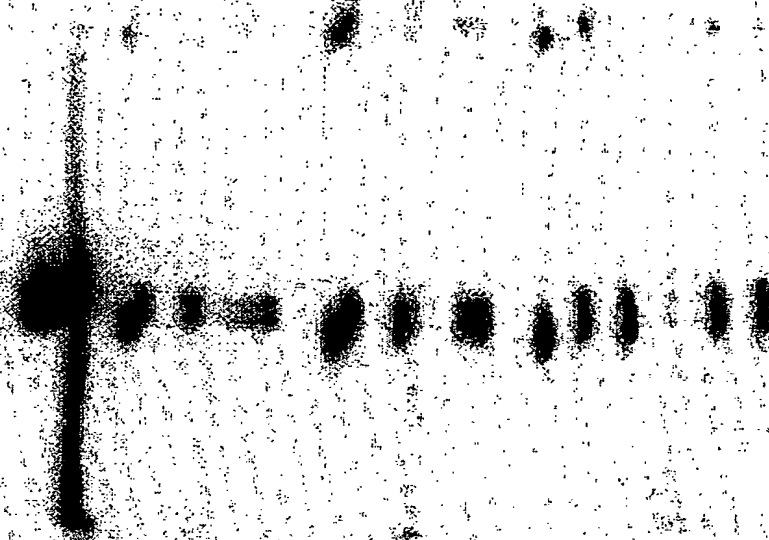


Fig. 2

AhS G H I J K
mM TSAT 0.1 1 10 100 0.1 1 10 100 0.1 1 10 100 0.1 1 10 100 0.1 1 10 100

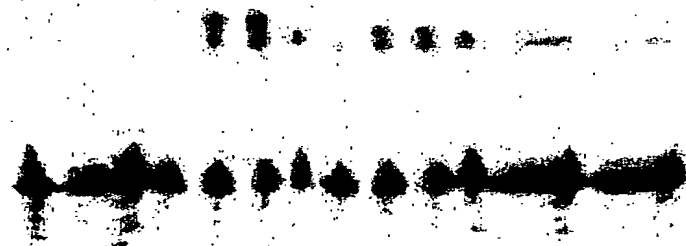


Fig. 3

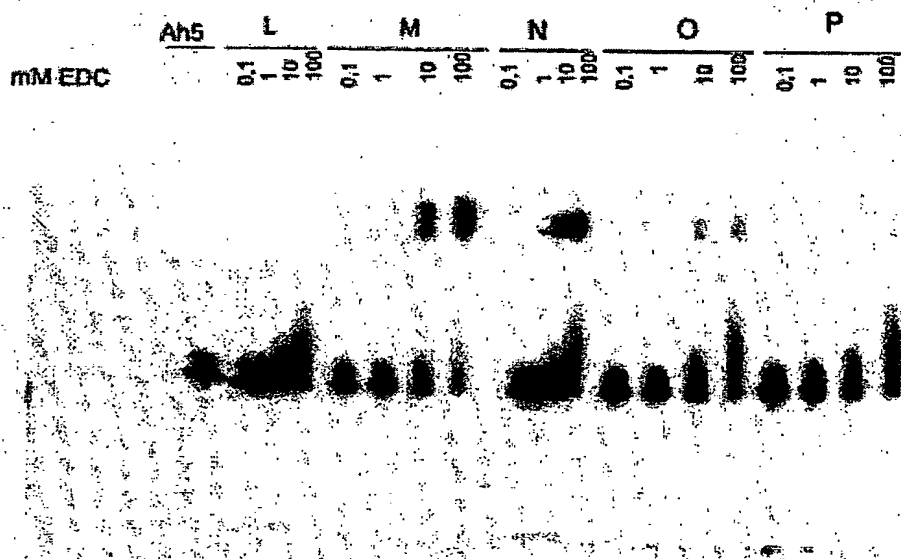


Fig. 4

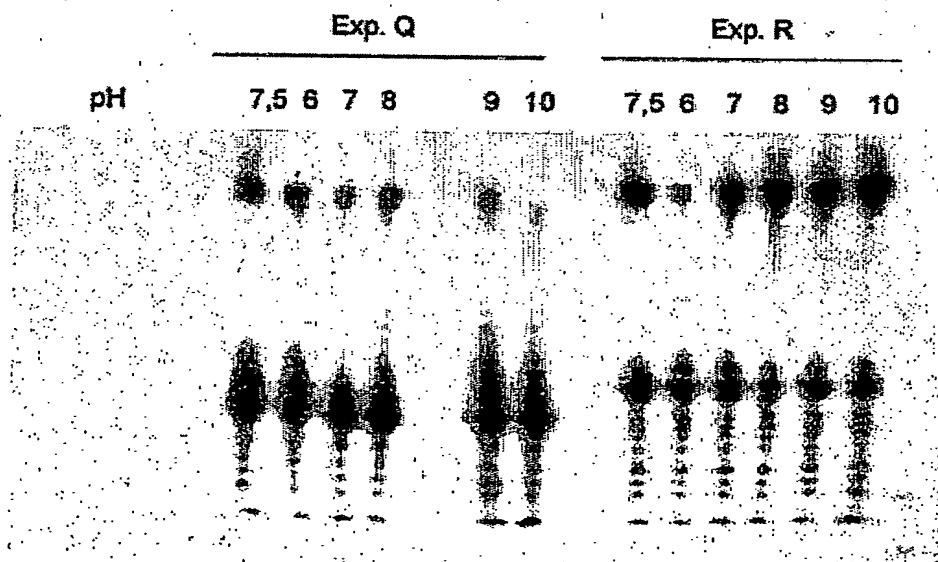


Fig.5

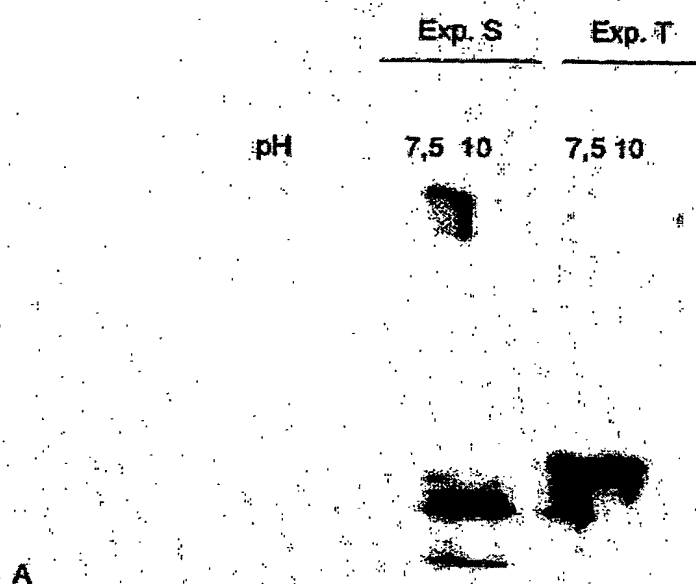


Fig. 6

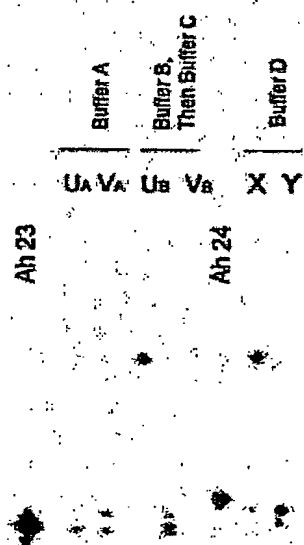


Fig. 7



Fig. 8

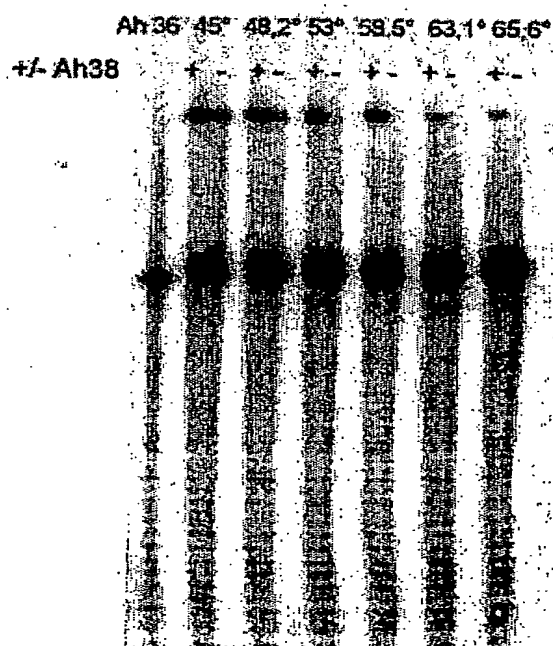


Fig. 9A

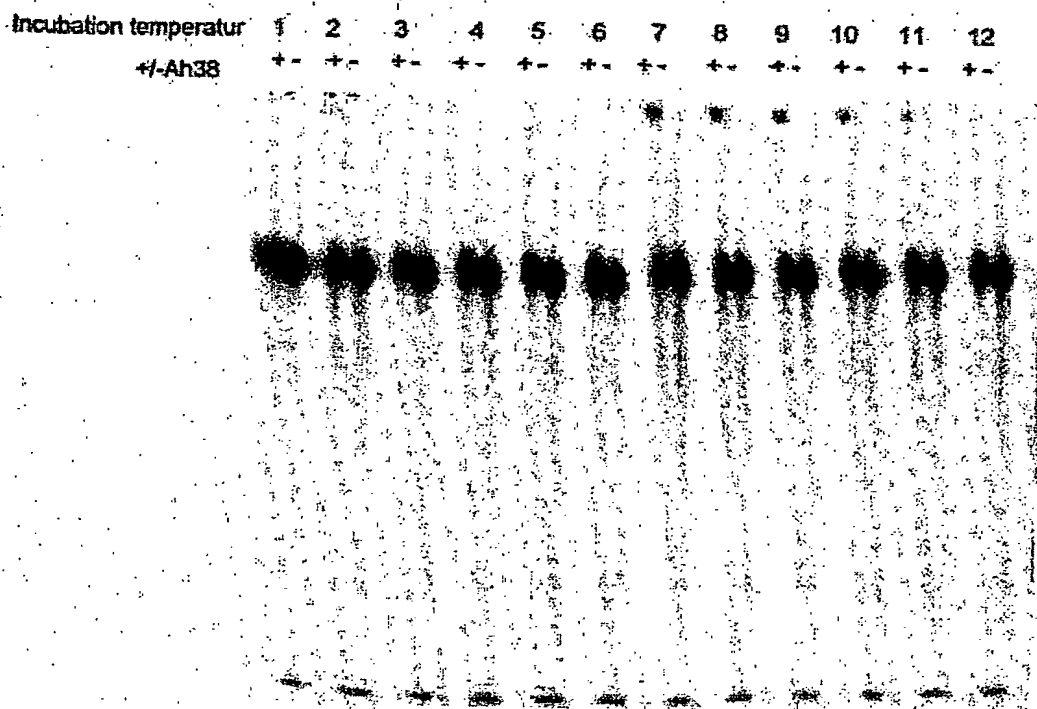


Fig. 9B

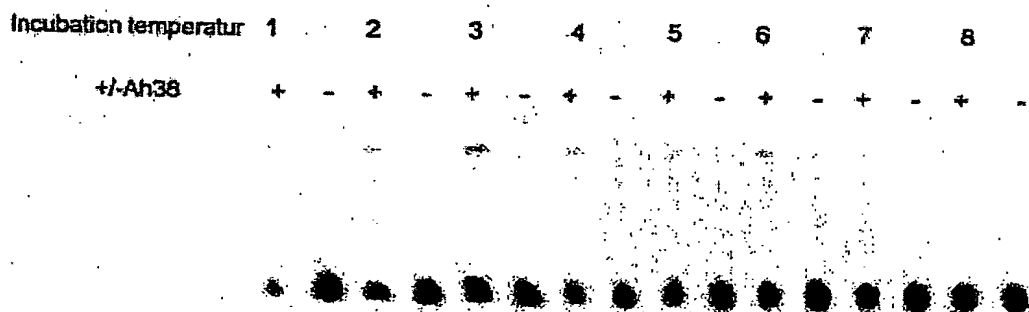


Fig.10A

	7,7 °C								15,4 °C								21,0 °C							
Experiment 10-	1	2	3	4	5	6	7	8	1	2	3	4	5	6	7	8	1	2	3	4	5	6	7	8



Fig. 10B

	26,0 °C								10 °C – 35 ° C (*99)							
Experiment 10-	1	2	3	4	5	6	7	8	1	2	3	4	5	6	7	8

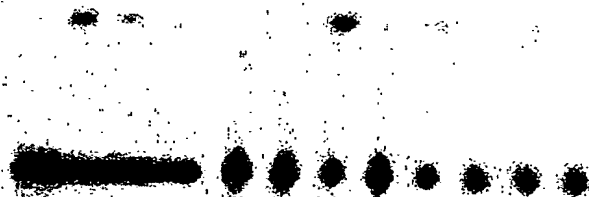
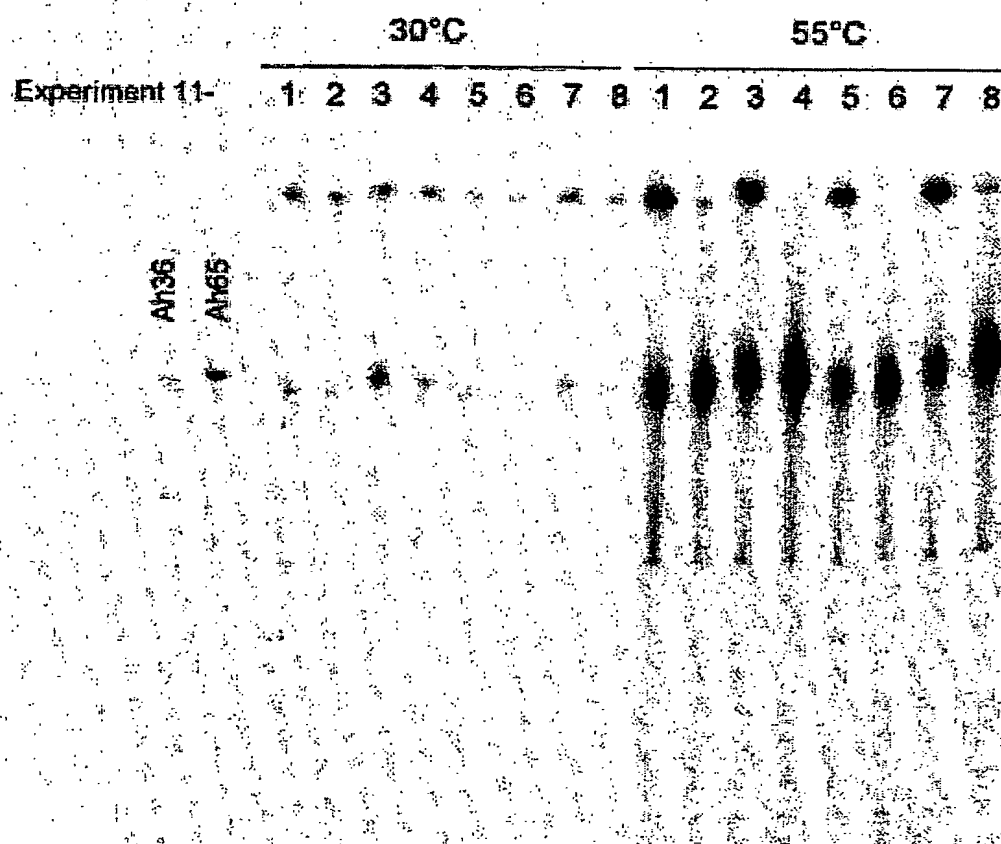


Fig.11



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Fig. 12

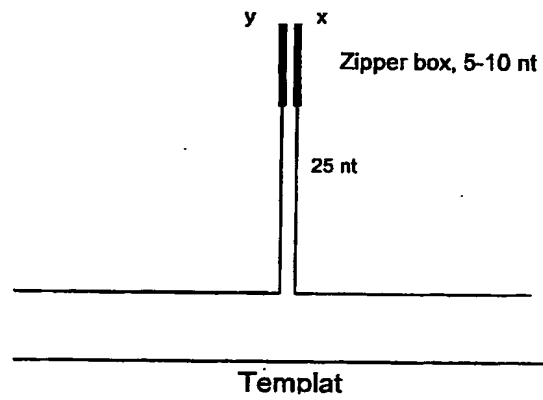
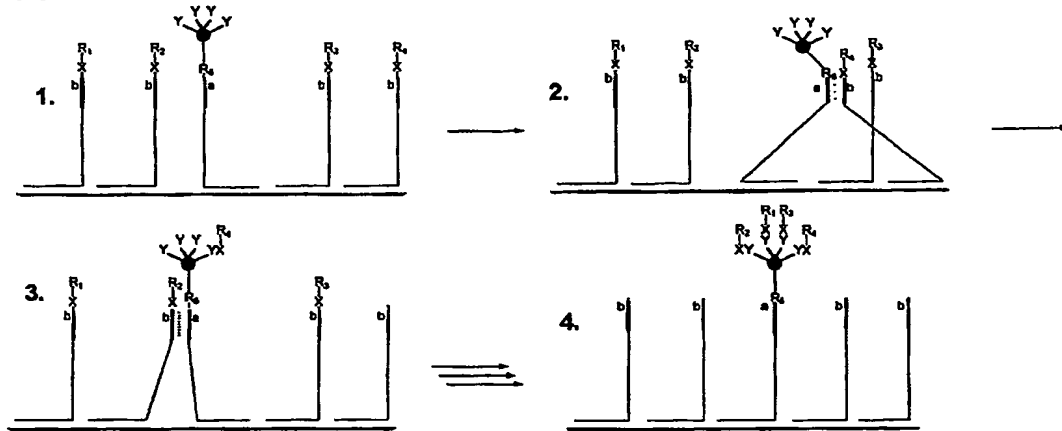


Figure 13

A.



B.

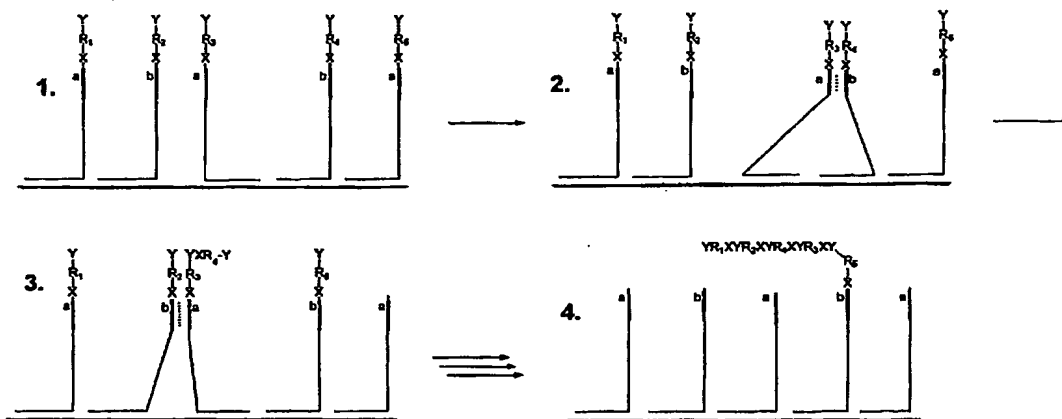


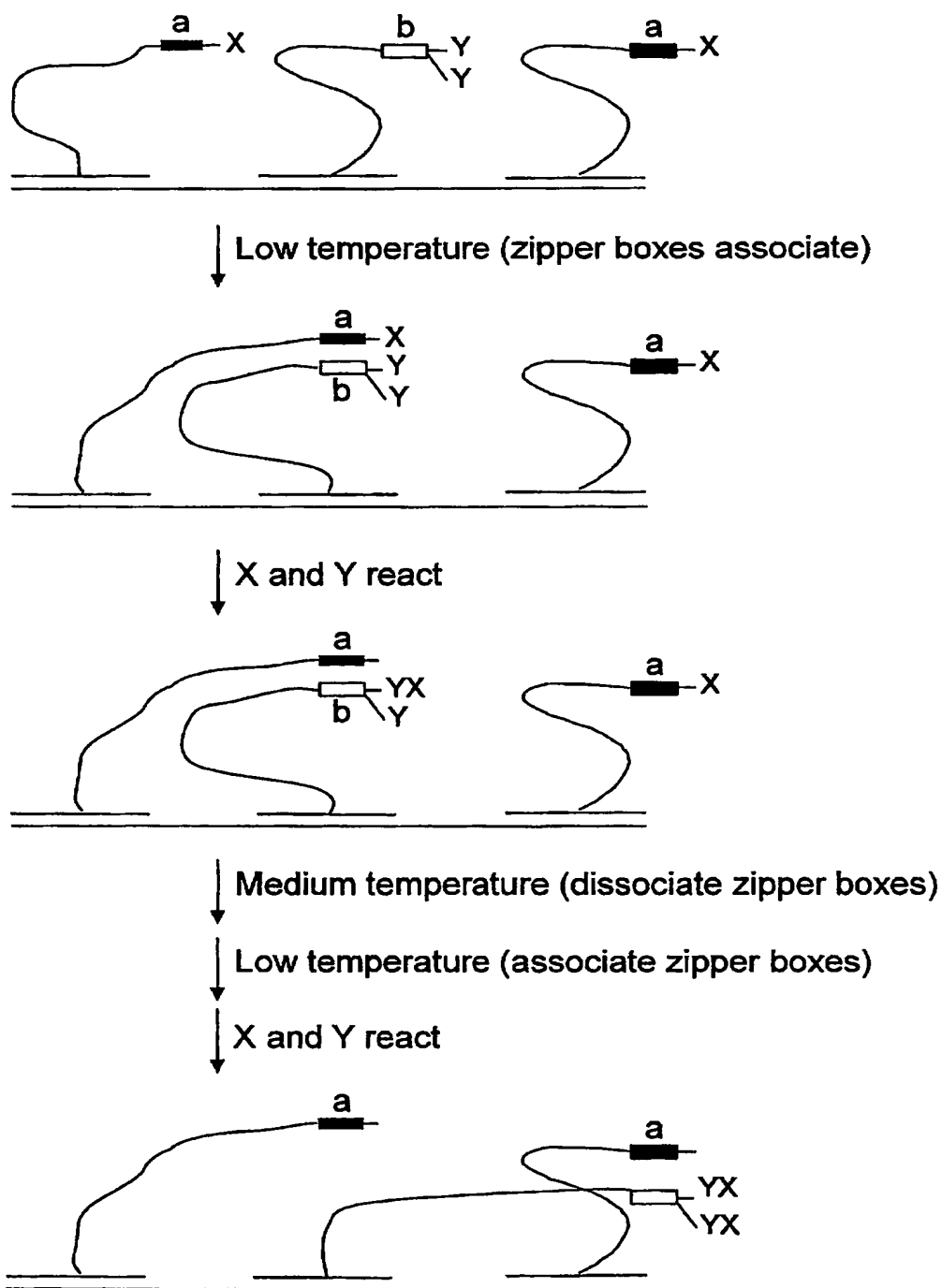
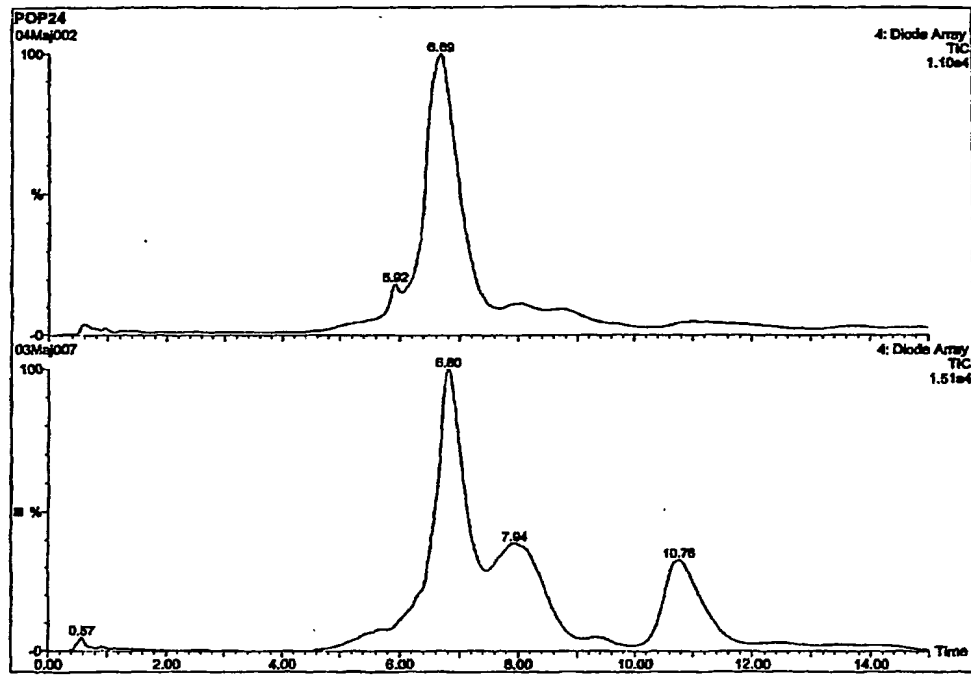
Figure 14 The Zipper box principle.

Fig. 15.



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